To Prime, or Not to Prime: That Is the Question

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Mitochondrial priming is regulated by the B-cell lymphoma 2 (BCL-2) family of proteins and determines a cell's "readiness" for apoptosis. A highly primed cell will undergo apoptosis more easily than an unprimed cell in response to apoptotic stimuli via the intrinsic apoptotic pathway. Priming can be measured via BH3 profiling, which uses BH3 peptides derived from the BH3 domain of pro-apoptotic BH3-only BCL-2 family members to provoke a response from viable mitochondria. BH3 profiling can be performed on tumor cells and can identify mechanisms a cell uses to evade apoptosis and anti-apoptotic dependency to the anti-apoptotic BCL-2 family members. Priming correlates with chemosensitivity of patients in multiple cancers. Therapeutics that enhances priming of patient tumor cells *ex vivo* could be used to aid therapeutic decisions for patients in the future.

Apoptosis is a form of physiological programmed cell death in multicellular organisms, and because of its functionality, it is tightly regulated (Wyllie 1997). Apoptosis acts as a barrier against tumorigenesis and, consequently, tumors select for mechanisms to resist apoptosis. Such mechanisms are hallmarks of cancer and are recognized to result in resistance to anticancer therapies (Hanahan and Weinberg 2000, 2011). There is a real need for new and improved therapies to overcome apoptotic resistance in cancer. The two major apoptotic pathways are the intrinsic (the focus of this review) and the extrinsic pathways. Both ultimately result in the activation of initiator caspases (cysteiny l aspartate specific protease; caspase-8 and -9) that in turn activate downstream effector caspases (caspase-3 and -7), triggering a caspase cascade (Li et al. 1997; Fadok et al. 2000; Savill and Fadok 2000).

INTRINSIC APOPTOSIS AND THE BCL-2 FAMILY

The intrinsic apoptotic pathway, once activated, results in mitochondrial outer membrane permeabilization (MOMP), usually the point of no return. MOMP causes the release of pro-apoptotic factors such as cytochrome *c* (Fig. 1) and SMAC (second mitochondrial derived activator of caspases) from the mitochondrial intermembrane space (Wang 2001). Once cytochrome *c* enters the cytosol, it interacts with APAF1 (apoptotic protease activating factor-1), the initiator procaspase-9, and ATP to form a holoenzyme known as the apoptosome. The formation of the apoptosome results in activation of caspase-9. Caspase-9 then cleaves and activates caspase-3 (Li et al. 1997). The activated caspase cascade causes cell-wide specific proteolysis, dysfunction, decrease in ATP pro-

duction, and remodeling of the cell surface with "molecular flags" such as externalization of phosphatidylserine. This allows the cell to be recognized by phagocytic cells like macrophages that then engulf the apoptotic cell (Fadok et al. 2000, 2001; Toda et al. 2012; Segawa et al. 2014). This efficient removal of dying cells allows biological "building blocks" to be recycled, an immune response to dead cells is contained, and damage to healthy cells in the vicinity of apoptotic cells is minimized, in contrast to necrosis and necroptosis (Li et al. 1997; Savill and Fadok 2000; Guerriero et al. 2011; Meng et al. 2016).

MOMP is regulated by the B-cell lymphoma 2 (BCL-2) family of proteins and can be activated by various mechanisms such as developmental cues, DNA damage, growth factor deprivation, nutrient deprivation, and treatment with a cytotoxic agent (Lovell et al. 2008; Youle and Strasser 2008; Fu et al. 2009; Spencer et al. 2009). BCL-2 family members can be grouped into three subfamilies, the pro-apoptotic effectors, the anti-apoptotic effector, and the pro-apoptotic BH3-only proteins (Fig. 1). The effector subfamily are multidomain proteins, containing BH (BCL-2 homology) 1, 2, 3, and 4 domains (Suzuki et al. 2000; Moldoveanu et al. 2006; Kvensakul et al. 2008). The members of this subfamily are BAK (BCL2-antagonist/killer), BAX (BCL-2-associated protein), and BOK (BCL2-related ovarian killer) (Youle and Strasser 2008). BAK is an integral mitochondrial protein found on the cytosolic side of the outer mitochondrial membrane (OMM). A prelude to MOMP after apoptotic stimuli is BAK undergoing a conformational change in the amino terminus revealing a newly exposed epitope (Griffiths et al. 1999). This leads to a second step activation in which BAK's BH1 domain is revealed and parallels the release of anti-apoptotic BCL-2 family members from BAK (Griffiths et al. 2001). In

Figure 1. Intrinsic apoptotic pathway. The intrinsic apoptotic pathway can be activated by various factors such as growth factor deprivation and cellular stress. Apoptotic signals result in an increase in active/available pro-apoptotic B-cell lymphoma 2 (BCL-2) family members (green, blue, and purple) and/or decrease in active/available anti-apoptotic BCL-2 family members (red) shifting the cell survival equilibrium toward apoptosis. This allows the pro-apoptotic effectors BCL-2-antagonist/killer (BAK) and BCL-2-associated protein (BAX) (purple) to be released and activated by pro-apoptotic BH3-only proteins BCL-2-interacting mediator of cell death (BIM) or BH3-interacting domain death agonist (BID) (green) resulting in BAX/BAK homo-oligomerization in the outer mitochondrial membrane, mitochondrial outer membrane permeabilization (MOMP), and apoptosis. *NOXA and PUMA (p53 up-regulated modulator of apoptosis) can act as activators. (Illustration made using Servier Medical Art.)

healthy cells, BAX is cytosolic (Hsu and Youle 1997; Wolter et al. 1997). In response to apoptotic stimuli, BAX undergoes a conformational change in the amino terminus resulting in BAX translocation to the OMM (Makin et al. 2001). On the OMM, activated BAK and BAX form homo-oligomer, creating pores and therefore MOMP (Fig. 1; Eskes et al. 2000; Korsmeyer et al. 2000; Wei et al. 2001). BAK and BAX are functionally redundant; however, loss of both prevents intrinsic apoptosis, showing that BAX and BAK are essential for MOMP (Lindsten et al. 2000). BOK is the least studied of the effector proteins and is primarily expressed in reproductive tissue (Hsu et al. 1997) but also in brain and myeloid cells (Ke et al. 2012). BOK has been shown to have redundant function with BAK and BAX in the hematopoietic system (Ke et al. 2015). In contrast to BAX and BAK, it may be primarily controlled by protein stability (Llambi et al. 2016).

The anti-apoptotic subfamily consists of the multidomain proteins BCL-2, BCL-xL (BCL-2-like protein 1, isoform long), BCL-w (BCL-2-like protein 2), MCL-1 (myeloid cell leukemia 1), and A1 (BCL-2-related pro-

tein A1, also known as BFL-1) (Fig. 1). These conserved proteins share homology in BH1-4 domains and are predominantly located in the OMM, but they are also present in the cytosol and endoplasmic reticulum (Lithgow et al. 1994; Pinton and Rizzuto 2006). The BCL-2 anti-apoptotic members prevent MOMP via antagonizing interactions with the pro-apoptotic BCL-2 family members (Chipuk et al. 2010). The BH1, -2, and -3 domains of the anti-apoptotic family members fold into a globular tertiary structure forming a hydrophobic groove on its surface. The hydrophobic groove provides a platform for the pro-apoptotic BCL-2 family members to interact via their BH3 domains (Sattler et al. 1997; Liu et al. 2003; Chonghaile and Letai 2008).

The pro-apoptotic BH3-only subfamily includes BAD (BCL-2-associated agonist of cell death), BID (BH3-interacting domain death agonist), BIK (BCL-2-interacting killer), BIM (BCL-2-interacting mediator of cell death), BMF (BCL-2-modifying factor), HRK (Harakiri), NOXA, and PUMA (p53 up-regulated modulator of apoptosis) (Chipuk et al. 2010). The BH3-only proteins are so-called because of their shared homology with BH3

		Pro-apoptotic BH3-only						
		Activators			Sensitizers			
		BIM	BID	BAD	BMF	HRK	NOXA*	PUMA*
Anti-apoptotic	BCL-2							
	BCL-xL							
	BCL-w							
	MCL-1							
	A1							

Figure 2. Specificity in binding of the pro-apoptotic BH3-only proteins to the anti-apoptotic BCL-2 family members. BH3-only proteins can be further divided into activator and/or sensitizers depending on their apoptotic properties. BH3-only peptides bind and antagonize specific anti-apoptotic family members. Pink indicates high-affinity binding and orange indicates poor/no binding (Letai et al. 2002; Certo et al. 2006; Deng et al. 2007). *NOXA and PUMA can act as activators. BIM, BCL-2-interacting mediator of cell death; BID, BH3-interacting domain death agonist; BAD, BCL-2-associated agonist of cell death; BMF, BCL-2-modifying factor; HRK, Harakiri; PUMA, p53 up-regulated modulator of apoptosis.

domains but little shared homology with the other BCL-2 family members (Aouacheria et al. 2005). BH3-only proteins show distinct specificities in their binding to the anti-apoptotic BCL-2 family members (Fig. 2) and can act as activators of BAK/BAX and/or sensitizer proteins (anti-apoptotic antagonists). BIM and BID are potent activators (Letai et al. 2002; Certo et al. 2006) that bind promiscuously to all the anti-apoptotic BCL-2 family members. BAD is a sensitizer and a BCL-2, BCL-w, and BCL-xL antagonist. HRK is a sensitizer that is a specific BCL-xL antagonist. BIK and BMF are sensitizers that antagonize BCL-2, BCL-w, BCL-xL, and MCL-1. Studies on the BH3 domain of PUMA (antagonist to all anti-apoptotic BCL-2 family members) showed no activator function (Letai et al. 2002; Kuwana et al. 2005; Certo et al. 2006). However, studies using full protein show that PUMA has activator properties (Kim et al. 2006). Double-knockout (DKO) BIM/BID mice do not recapitulate the BAK/BAX DKO mice phenotype, but BIM/BID/PUMA triple-knockout mice phenocopy BAK/BAX DKO mice (Ren et al. 2010; Du et al. 2011), suggesting that PUMA acts as an activator. NOXA is a sensitizer, a specific MCL-1 antagonist, that has been shown more recently to have an activator function (Du et al. 2011).

PRIMING

MOMP is a switch-like event that is regulated by the balance of pro- and anti-apoptotic proteins. One can consider MOMP like a cliff and imagine that some cells are closer to the cliff's edge than others. The cells proximity to this cliff's edge would carry with it significant physiologic consequences, including increased likelihood of commitment to cell death following toxic perturbations. We consider cells that are close to the cliff's edge to be relatively highly "primed" for apoptosis, whereas those far from the cliff's edge to be relatively "unprimed." It is important to discuss how priming can be measured so that primed and unprimed cells can be distinguished and what the molecular basis of this priming is. Priming (also referred to as mitochondrial priming or apoptotic priming) is also regulated by the BCL-2 family of proteins. The

balance between the BCL-2 anti-apoptotic and pro-apoptotic family members and the respective availability of their interaction sites determine how primed a cell is and in turn whether MOMP will occur. Priming is relative; the more primed a cell becomes, the lower the apoptotic threshold and tolerance for apoptotic stimuli (Fig. 3). Like apoptosis, priming is affected by a number of physiological conditions such as growth factor deprivation, changes in metabolism, or genetic aberrations (Certo et al. 2006). Drug treatment can enhance priming, proving advantageous in treating cancer (discussed in detail later).

BH3 profiling was developed as a functional tool to measure priming experimentally (Letai et al. 2002; Certo et al. 2006; Ryan et al. 2010). The assay uses synthetic BH3 peptides derived from the BH3 domain of the pro-apoptotic BH3-only proteins to provoke a response from the mitochondria. Priming is measured in terms of MOMP induced by a BH3 peptide. The different platforms of BH3 profiling measure a surrogate for MOMP, such as loss of JC-1 red fluorescence or cytochrome *c* released from the mitochondria (Ryan and Letai 2013; Ryan et al. 2016).

As discussed previously BH3-only proteins can either be promiscuous or selective in their affinity for anti-apoptotic BCL-2 family members. Therefore, the specificity of BH3 peptides to the anti-apoptotic BCL-2 family members allows BH3 profiling to differentiate between promiscuous overall priming (determined by increased MOMP after BIM, BID, or PUMA exposure) or a more selective measure of anti-apoptotic dependency. For example, if a BH3 profile revealed that the sensitizer BH3 peptide HRK caused MOMP, this would suggest that these cells are likely dependent on BCL-xL because HRK is a selective antagonist for BCL-xL (Letai et al. 2002; Certo et al. 2006).

When a carefully selected panel of BH3 peptides are used, BH3 profiling can also reveal what apoptotic block cancer cells are likely using to evade apoptosis and thus provides a measure of how primed that cell is (Fig. 4; Deng et al. 2007). For instance, a cell that evades apoptosis via down-regulation of pro-apoptotic BH3-only activators will only respond to activator peptides in the

Figure 3. Primed versus unprimed cellular response to apoptotic stimuli. Priming is relative; therefore, a primed cell has a relatively lower apoptotic threshold than an unprimed cell. An apoptotic assault such as treatment with a BH3 mimetic is likely to cause mitochondrial outer membrane permeabilization (MOMP) on a primed cell but not an unprimed cell. MOMP with a red $\times$ indicates no MOMP occurred. MOMP with a green $\checkmark$ indicates MOMP occurred.

BH3 profiling assay (Fig. 4A [green bars]). The inability of either activator or sensitizer BH3 peptides to induce cytochrome *c* release by BH3 profiling would suggest a lack of functional BAX/BAK in those cells (Fig. 4B). If both activator and sensitizer BH3 peptides could induce cytochrome *c* release, then a mechanism of evading apoptosis via up-regulation of anti-apoptotic BCL-2 family members would be suggested (Fig. 4C [green and blue bars]). This type of cell would be classed as primed because all the components to induce apoptosis are present, but they are constantly neutralized by the anti-apoptotic BCL-2 family members (Deng et al. 2007).

PRINCIPLES OF BH3 PROFILING

The BH3 domain was first described in BIK more than 20 years ago (Boyd et al. 1995) and is essential for interactions among BCL-2 members (Zha et al. 1996; Sattler et al. 1997). Short synthetic BH3 peptides were used to investigate BCL-2 family member interactions and showed that BIM and BID BH3 peptides cause BAX and BAK oligomerization (Letai et al. 2002; Certo et al. 2006). At this point, BH3 profiling was performed on isolated mitochondria using an enzyme-linked immunoabsorbent assay (ELISA)-based method to measure

cytochrome *c* released from the mitochondria, after exposure to a fixed concentration of BH3 peptide. Released cytochrome *c* is used as a surrogate of MOMP (Letai et al. 2002; Certo et al. 2006). The early method of BH3 profiling had limitations such as lack of incorporation cell surface markers, low throughput, and requirements for a relatively large number of cells as starting material (10^8 – 10^9 cells). Limitations were addressed and BH3 profiling on whole cells was developed, reducing the number of cells needed by 100-fold. The current BH3 profiling techniques most commonly used in the Letai laboratory are plate-based JC-1 BH3 profiling (Ryan and Letai 2013) and iBH3 (intracellular BH3) (Ryan et al. 2016).

For BH3 peptides to engage with the mitochondria of viable cells, the plasma membrane must be permeabilized to allow the diffusion of the BH3 peptides into the cell. Digitonin is a glycoside that precipitates cholesterol, which is relatively more abundant in the plasma membrane than organelles. A low concentration of digitonin (5–25 $\mu\text{g/mL}$), lightly permeabilizes the plasma membrane but keeps organelles intact, allowing BH3 peptides to interact with intact mitochondria. Once MOMP occurs the cell is committed to intrinsic apoptosis and therefore MOMP is the end point for BH3 profiling (Ryan and Letai 2013; Ryan et al. 2016). Activation of MOMP causes depolarization of the transmembrane mitochondrial

Figure 4. Anti-apoptotic mechanisms in cancer cells. Cancer cells can evade apoptosis via three main mechanisms involving the BCL-2 family, and BH3 profiling can distinguish these mechanisms via induction of mitochondrial outer membrane permeabilization (MOMP) after exposure to individual activator or sensitizer BH3 peptides. Down-regulation of pro-apoptotic BH3-only activators would evade apoptosis because pro-apoptotic effectors BCL-2-associated protein (BAX)/ BCL-2-antagonist/killer (BAK) cannot be activated in the absence of activators, and therefore no MOMP. BH3 profiling on this type of cell would be determined because activator peptides (green bars) would induce MOMP, but sensitizer peptides (blue bars) would not (A). The second mechanism of evading apoptosis is through inactivation or down-regulation of BAX/BAK, because without BAX/BAK, there is no MOMP. BH3 profiling on a cell that uses this block would be determined when none of the BH3 peptides (activators or sensitizers) cause MOMP (B). The final mechanism to evade apoptosis is via up-regulation of anti-apoptotic BCL-2 family members, as this would neutralize any pro-apoptotic effectors or BH3-only proteins preventing MOMP. BH3 profiling on cells that use this block would be determined when both activator and sensitizer BH3 peptides cause MOMP (C) (Deng et al. 2007). (Illustration made using Servier Medical Art.)

potential ($\Delta\Psi_m$). JC-1 is a fluorescent dual-emission mitochondrial intermembrane probe that fluoresces red (JC-1 aggregates) when concentrated in the matrix of a healthy, polarized mitochondria or green when $\Delta\Psi_m$ has depolarized because of MOMP (JC-1 monomers). Once cells have been permeabilized and treated with a fixed concentration of BH3 peptide, loss of JC-1 red fluorescence caused by $\Delta\Psi_m$ depolarization, is used as a surrogate for the end point MOMP in plate-based JC-1 BH3 profiling (Fig. 5). This BH3 profiling platform using JC-1 allows real-time kinetic measurements of loss of mitochondrial integrity (Ryan and Letai 2013).

If the cell population is homogenous, such as in cell lines or a cell-sorted population, then a plate-based approach can provide high throughput for the lowest cost. However, if BH3 profiling is performed on a mixed population of cells, such as a primary sample, the iBH3 approach would be more useful. Intercellular BH3 is a flow cytometry-based assay that measures retained cytochrome *c* as a surrogate to MOMP. Primary samples are

dissociated using enzymatic and mechanical forces to break down the extracellular matrix, leaving a single-cell suspension. Cells can be labeled for cell surface markers to identify cell subpopulation of interest. Labeled cells are then permeabilized with digitonin, exposed to BH3 peptides of known concentration for a given time, and fixed with formaldehyde. Cells are then incubated with a cytochrome *c* antibody to detect retained cytochrome *c* (Fig. 5). The cell fixation step provides an advantage for iBH3 because infectious primary samples including HIV can be handled; also, samples can be analyzed weeks after fixation (Ryan et al. 2016).

BH3 MIMETICS TO TARGET THE BCL-2 FAMILY

Alterations in the function and expression of the BCL-2 family of proteins contribute to cancer progression, highlighting their potential as rational drug targets. Compared

Figure 5. BH3 profiling platforms. Plate-based JC-1 BH3 profiling versus iBH3 (intracellular BH3).

to healthy cells, cancer cells have increased apoptotic signaling caused by factors such as oncogene activation and changes in the microenvironment (Hanahan and Weinberg 2000, 2011). Cancer cells become reliant on anti-apoptotic signaling to survive, known as “oncogene addiction,” and drugs have been developed that target and exploit this. This led to the development of small-molecule antagonists that can block interactions between anti-apoptotic and pro-apoptotic BCL-2 family members, known as BH3 mimetics (Oltersdorf et al. 2005; Souers et al. 2013).

BH3 mimetics compete with pro-apoptotic BCL-2 family proteins for binding in the BH3 binding site of anti-apoptotic proteins. Their binding to anti-apoptotic proteins releases the pro-apoptotic BCL-2 family members to induce MOMP (Fig. 3). The first BH3 mimetic, ABT-737, was developed in 2005 (AbbVie) and is functionally analogous to the BH3 domain of BAD, binding BCL-2, BCL-xL, and BCL-w (Oltersdorf et al. 2005). Preclinical studies showed that ABT-737 has single-agent activity in some small cell lung cancer (SCLC) and lymphoma cell lines as well as in primary chronic lymphocytic leukemia (CLL) and lymphoma cells (Oltersdorf et al. 2005; Deng et al. 2007; Tahir et al. 2007; Del Gaizo Moore et al. 2008; Mason et al. 2008). ABT-737 was also found to act synergistically with conventional chemotherapeutic agents and targeted therapies in a variety of cancer types (Premkumar et al. 2012; Gardner et al. 2014; Potter et al. 2014, 2016). Cells that rely on BCL-2, BCL-w, or BCL-xL for survival are particularly sensitive to ABT-737. ABT-737 is unable to interact with MCL-1

and, thus, expression of this BCL-2 family member is a resistance factor for ABT-737 efficacy (Vogler et al. 2009). Indeed, ABT-737 sensitization caused by other drugs or the tumor microenvironment is often associated with MCL-1 down-regulation (Harrison et al. 2011; Gardner et al. 2014).

A clinical derivative of ABT-737 is navitoclax (ABT-263), an orally bioavailable BH3 mimetic (Tse et al. 2008). Navitoclax has shown promise in the phase I setting, in which biomarkers of apoptotic cell death were detected in patients treated with navitoclax (Gandhi et al. 2011). It has been tested in phase I/II clinical trials in SCLC, ovarian, acute lymphocytic leukemia (ALL), and CLL patients as a single agent and in combination with chemotherapy (clinicaltrials.gov). Navitoclax showed promising single-agent efficacy in phase II clinical trials in CLL (Roberts et al. 2012) but not in SCLC (Gandhi et al. 2011). Combining navitoclax with targeted agents such as MEK (MAPK/ERK kinase), mTOR (mammalian target of rapamycin), or PI3K (phosphoinositide 3-kinase) inhibitors appears to increase the potency of navitoclax in preclinical models in NSCLC (non-small cell lung cancer), pancreatic cancer, and SCLC (Tan et al. 2013; Faber et al. 2014, 2015; Potter et al. 2016), although the clinical impact of these findings remains to be determined. Thrombocytopenia is a dose-limiting toxicity of navitoclax, caused by the on-target inhibition of BCL-xL, as platelets are BCL-xL-dependent (Tse et al. 2008; Schoenwaelder et al. 2011). This led to the development of a more specific BCL-2 antagonist (Souers et al. 2013).

Venetoclax (ABT-199) is a BCL-2-selective BH3 mimetic that does not cause thrombocytopenia. Eleven years after the first BH3 mimetic was developed, venetoclax (co-developed by AbbVie and Genentech), was approved by the FDA in CLL patients with 17p deletion after receiving at least one prior treatment (Anderson et al. 2016; Roberts et al. 2016). Venetoclax is currently in clinical trials in other hematological malignancies (acute myeloid leukemia [AML] and lymphoma) (clinicaltrials.gov). It was shown that the Tec family kinase inhibitor, ibrutinib, sensitized to venetoclax in CLL, in which ibrutinib has also been approved for second-line treatment. Sensitization is via inhibition of the Tec family kinase, BTK, and was associated with a decrease in MCL-1 and BCL-xL levels (Cervantes-Gomez et al. 2015). In another study in CLL, venetoclax-resistant cells were sensitized to venetoclax when venetoclax was combined with the BEZ235 (dual PI3K/mTOR inhibitor), associated with MCL-1 down-regulation due to PI3K pathway inhibition (Choudhary et al. 2015). As of this writing, the first clinical trial of an MCL-1 inhibitor (AMG 176), made by Amgen, is under way in relapsed or refractory myeloma (clinicaltrials.gov).

BH3 PROFILING AS A PREDICTOR FOR CHEMOSENSITIVITY

For the past 50 years, conventional chemotherapy has been the mainstay of cancer therapy, curing millions of people worldwide. In recent years, a more targeted approach has further improved therapeutic outcome in some cancers. However, not all cancer patients respond to chemotherapy. BH3 profiling has been used in many studies, in different cancer types, to successfully predict chemosensitivity and clinical outcome.

In multiple myeloma, serum levels of the monoclonal protein (M protein) are measured as an indication of tumor burden (Durie et al. 2006). BH3 profiling using BMF and PUMA peptides to measure priming was performed on 51 multiple myeloma patient before treatment. This study showed that priming correlated with a decrease in M protein and therefore tumor burden after chemotherapy. Priming also correlated with better clinical outcome for patients after chemotherapy. Highly primed patients had a longer progression-free survival than the poorly primed patients (Ni Chonghaile et al. 2011).

Pediatric ALL has a long-term survival rate of 80%–90% compared to adult ALL, with a survival rate of 40%, suggesting that age is a factor in prognosis. BH3 profiling on both pediatric and adult ALL samples revealed that pediatric ALL is more primed than adult. This increased priming observed in pediatric ALL could explain the superior response rate to chemotherapy. A further look into patient's clinical response showed that patients who responded and then relapsed were less primed compared to patients that responded but did not relapse (Ni Chonghaile et al. 2011).

Two studies in AML showed that priming correlated with clinical response to chemotherapy. In one study BH3

profiling on malignant myeloblasts from patients' bone marrow before any therapy revealed that patients who had complete remission were more primed than those that did not (Ni Chonghaile et al. 2011). In a second study, priming of malignant myeloblasts from AML patients (measured by BH3 profiling with BIM peptide) correlated with response to chemotherapy. Patients with highly primed myeloblasts had a better clinical outcome to chemotherapy compared to patients with poorly primed myeloblasts (Vo et al. 2012).

BH3 profiling was performed on CLL cells from patient blood before any chemotherapy. The promiscuous BH3 peptide BIM was used to measure priming. Patients were then treated and followed for clinical response. Patients who had either a partial or complete response to therapy were significantly more primed than patients that had stable or progressive disease. In CLL, priming successfully predicts chemosensitivity and could be used as a predictive biomarker for response (Davids et al. 2012).

BH3 profiling as a predictor of chemosensitivity has also been shown in solid tumors. In ovarian cancer, BIM, PUMA, and BMF peptides revealed that highly primed tumors correlated with superior progression-free survival (Ni Chonghaile et al. 2011).

To determine whether priming is responsible and not just correlative for chemosensitivity, a study investigated mechanisms to enhance priming to assess whether this increased sensitivity to chemotherapy agents. As previously mentioned, BH3 mimetics directly target the anti-apoptotic BCL-2 family members and therefore may increase priming. BH3 mimetics treatment reduces anti-apoptotic binding site availability and free activators to activate BAK and/or BAX, lowering the apoptotic threshold (Fig. 3). The myeloid leukemia cell line K562 was treated with ABT-737 and the effect on chemosensitivity was assessed. ABT-737 caused increased sensitivity to doxorubicin, vincristine, and etoposide, suggesting that priming is a determinant of chemosensitivity (Ni Chonghaile et al. 2011).

PRIMING OVER TIME

The difference in pediatric and adult priming observed in ALL suggested possible age-related differences in priming of normal tissues as well. Priming in young and adult mice was investigated using BH3 profiling. This revealed that in healthy young mice, brain, heart, and kidney cells were extremely sensitive to BH3 peptides and were highly primed. In contrast, mitochondria in the tissue of healthy adult mice were completely insensitive to BH3 peptides. Intrinsic apoptosis could only be triggered in these cells with the addition of exogenous BAX protein, suggesting that healthy, adult brain, heart, and kidney tissue are incompetent for apoptosis. This was confirmed with immunoblotting levels of BCL-2 family members in a series of developmental time points from prenatal to postnatal into adulthood. BAX and BAK levels were dramatically down-regulated during postnatal development to nearly undetectable levels by adulthood.

Without BAX and BAK, cells cannot undergo MOMP and are therefore incompetent for intrinsic apoptosis. BH3 profiling in healthy, human brain tissue confirmed that adult brain cells are less primed than child brain cells, strengthening the findings in mice. BH3 profiling in young and adult spleen and bone marrow revealed that high levels of priming continue into adulthood, emphasizing the organ-specific regulation of apoptotic machinery (Sarosiek et al. 2015).

MECHANISM TO ENHANCE PRIMING

Priming correlates with clinical response to therapy; therefore, mechanisms to enhance priming would be beneficial to the patient. Mechanisms to enhance priming led to the development of dynamic BH3 profiling, in which tumor cells are pretreated with a drug and the effect on priming is measured using BH3 profiling. Dynamic BH3 profiling uses a functional approach to precision medicine; an increase in priming compared to untreated control is referred to as delta priming. Dynamic BH3 profiling integrates net changes in BCL-2 family members after treatment with a therapy, measuring total proapoptotic signaling. Delta priming distinguishes cells to be killed by a therapy and therefore predicts therapies that will have a cytotoxic effect before the event in various models and on patient samples. Delta priming can be used to predict a therapy's cytotoxic response in vitro, in vivo, and importantly in the clinic (Montero et al. 2015).

CONCLUSION

Predicting the most efficacious drug for a patient is ideal. Unless there is specific knowledge of that patient's tumor available, such as actionable driver mutations identified through tumor genotyping, dynamic BH3 profiling could be used to overcome imperfect knowledge and predict therapies that enhance priming. As well as identifying individual compounds that enhance priming, dynamic BH3 profiling can also reveal combination regimens of said compound with BH3 mimetics, improving therapeutic outcome for patients. The goal of precision medicine is to match a patient to the best treatment available. Genomics approaches dominate precision medicine although they currently benefit only a minority of cancer patients. We anticipate that functional precision medicine approaches, including dynamic BH3 profiling, by measuring what happens when the drug of interest contacts the tumor cell of interest, will play a major role in the future of precision medicine in cancer.

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